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Isolation of a Glucan-Binding Domain of Glucosyltransferase (1,6-α-Glucan Synthase) from *Streptococcus sobrinus*

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A glucan-binding domain of 1,6-α-glucan synthase (dextransucrase) (GTF-S) was isolated from a trypsin digest of the *Streptococcus sobrinus* enzyme. The large 60.5-kilodalton peptide had an affinity for dextran comparable to that of the native enzyme, but had no glucan synthesis activity. The domain was produced in high yield compared with other large cleavage products, which allowed easy purification by size exclusion high-pressure liquid chromatography and affinity chromatography. Two other proteases (mouse submaxillaris protease and lysyl endopeptidase) with specificities similar to trypsin generated a distribution of GTF-S peptides that was also greatly enriched in the glucan-binding peptide. Proteases with markedly different specificities (chymotrypsin and *Staphylococcus aureus* V8 protease) produced a family of peptides with some evidence of the glucan-binding domain but in far lower yield. The tertiary structure of the domain was critical to its resistance to proteolysis; heat denaturation of GTF-S before trypsin digestion resulted in cleavage of the enzyme to small limit peptides leaving no evidence of the glucan-binding domain. The amino acid composition of the peptide was very similar to that of the native enzyme. The common occurrence of proteases in oral streptococcus cultures and reports of glucosyltransferase degradation during purification and storage raises the possibility that some accounts of glucan-binding receptors are peptides derived from glucosyltransferase. Kinetic implications of a glucan-binding domain are discussed.

The cariogenicity of *Streptococcus sobrinus* and related oral streptococci is due in part to a family of glucosyltransferases (GTFs) released by this bacterium. The enzymes use sucrose to synthesize dental plaque polysaccharides which help mediate bacterial attachment to the tooth surface (11–13). The GTFs fall into two groups: those that synthesize a water-soluble, primarily α-1,6-linked glucan (GTF-S) and those that synthesize a water-insoluble, primarily α-1,3-linked glucan (GTF-I). In reality, the distinction among GTFs is more complex and includes enzymes which vary in glucan affinity and insertion of glucan branches (19).

Our understanding of GTF protein structure is very limited, amounting to knowledge of the enzyme mass (about 160 kilodaltons [kDa]) (19) and amino acid composition (7). There remain significant questions on the relation of GTF structure to catalysis, immunochemistry, and bacterial virulence. Given the similarities among these enzymes, there are likely many shared structural features, although immunological and biochemical differences have been clearly identified (19).

Large proteins like GTF are commonly composed of domains (14). The structural integrity of a domain can be highly dependent on other segments of the protein or, in some instances, can be structurally independent and capable of maintaining conformation separate from the protein. In the latter case, there is a potential to separate the domain from the protein and leave domain function intact as, for example, occurs with immunoglobulins (20).

We looked for evidence of domain structure in GTF by examining peptide products of proteolytic cleavage. Both GTF-S and GTF-I showed clear, reproducible digestion patterns leaving some large protein fragments undigested. GTF-S, in particular, had one predominant fragment after mild trypsin digestion which could be readily purified. The fragment did not catalyze glucosyl transfer, but did bind dextran with an affinity comparable to that of native GTF-S. These characteristics suggest that the peptide is a structural domain of GTF which functions in glucan binding.

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MATERIALS AND METHODS

Reagents. [U-^{14}C]sucrose was obtained from ICN Biochemicals (Cleveland, Ohio). Tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (222 U/mg) was from Organon Teknika (Malvern, Pa.), *Staphylococcus aureus* V8 protease (500 to 700 U/mg) and mouse submaxillaris protease (250 to 300 U/mg) were from Pierce Chemical Co. (Rockford, Ill.), lysyl endopeptidase was from Wako Chemicals USA (Dallas, Tex.), and chymotrypsin (57 U/mg) was from Sigma Chemical Co. (St. Louis, Mo.). *Streptococcus mutans* 6715 (serotype g) was purchased from American Type Culture Collection (Rockville, Md.).

Bacterial growth and enzyme purification. *S. sobrinus* (*S. mutans* 6715 [serotype g]) was purchased from American Type Culture Collection and cultured as previously described (18). Briefly, bacteria were grown in brain heart infusion (Difco Laboratories, Detroit, Mich.) and then passed on mitis salivarius agar and transferred to a chemically defined medium developed by Terleckyj et al. (24). Bacteria were generally cultured in 40-liter volumes. After approximately 30 h of culture with periodic pH adjustments, bacteria were separated from the medium with a Pellicon filtration apparatus (Millipore Corp., Bedford, Mass.). Cell-free medium was concentrated about 40-fold with a 10,000-molecular-weight-cutoff membrane on the same apparatus. GTF-S and GTF-I were purified by using an agarose-based affinity resin with covalently bound Dextran T-10. After approximately 1 liter of concentrated medium was loaded and the column was washed with 500 ml of 0.1 M sodium phosphate buffer containing 0.5 M guanidine hydrochloride (GndHCl) (pH 7.0), GTF-S and GTF-I were eluted with a 0.5 to 4.0 M GndHCl gradient in the same buffer (18). Purified GTF-S and GTF-I were homogeneous by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had respective activities of approximately 35 and 22 μmol/min per mg (initial velocity in 10 mM sucrose–0.05 mM Dextran T-10–0.05 M sodium phosphate-citrate buffer, pH 6.0, 37°C).

Gel electrophoresis. Discontinuous SDS-PAGE was performed by the method of Laemmli (15). The separating gel was 12% acrylamide poured in a vertical slab gel electrophoresis apparatus (7 cm by 8 cm by 0.75 mm; Mighty Small II; Hoefer Scientific Instruments, San Francisco, Calif.). Analytical samples were dialyzed against water (sample-water, 1:1,000, vol/vol) for 24 h with one water change, and then 15 to 30 μg was dried on a Savant Speed Vac centrifuge and reconstituted in 10 μl of solubilizing buffer. Electrophoresis was performed at 100 V and 4°C. Proteins were stained with Coomassie blue.

Protease digestion. Approximately 30 μg of GTF was digested with TPCK-trypsin, lysyl endopeptidase, or mouse submaxillaris protease in 200 μl of 0.2 M NH_4HCO_3 (pH 8.0) at 37°C. Protease/GTF (wt/wt) ratios were 1:100, 1:1,000, 1:5,000, 1:10,000, and 1:100,000. Preparative analyses used 1.0 mg of GTF in 2.5 ml of buffer. Digestion was halted after 20 h in one of two ways: (i) samples used for gel electrophoresis were boiled for 2 min; (ii) samples examined for dextran-binding ability were brought to 0.02% in SDS to halt trypsin and trypsinlike activity and then chromatographed or frozen. Digestion with chymotrypsin and *S. aureus* V8 protease included 0.02% SDS to eliminate the effects of any contaminating trypsinlike proteases.

Purification of glucan-binding peptide from GTF-S. Approximately 1.0 mg of GTF-S in 2.5 ml of NH_4HCO_3 buffer was digested with TPCK-trypsin at 1:5,000 (wt/wt) for 20 h at 37°C, and the reaction was stopped by bringing the mixture to 0.02% SDS. The digest was concentrated to 100 μl on a Savant centrifuge and then chromatographed on a BioSil TSK-250 gel filtration high-pressure liquid chromatography (HPLC) column (7.5 by 300 mm; Bio-Rad Laboratories, Richmond, Calif.) eluted with 0.05 M sodium phosphate (pH 7.0) at 1 ml/min. Elution was monitored at 280 nm. Fractions containing the glucan-binding peptide were identified by SDS-gel electrophoresis. Approximately 0.5 mg of glucan-binding peptide was recovered from the TSK-250 column. Part of the sample (200 μg) was loaded on an affinity column (9 by 23 mm) (18) equilibrated in 0.5 M GndHCl with 0.1 M sodium phosphate (pH 7.0) and eluted at 1 ml/min in the initial buffer with a gradient of 0.067 M GndHCl per min.

Analytical methods. Enzyme activity was measured with 10 mM [U-^{14}C]sucrose and 0.05 mM Dextran T-10 in 0.05 M phosphate-citrate buffer (pH 6.0) at 37°C. Initial velocities were measured by monitoring the production of [^{14}C]glucan as previously described (18). Protein was measured by a modified *o*-phthalaldehyde method described by Viets et al. (26) with bovine serum albumin as a standard. Samples for amino acid analysis were hydrolyzed in 6 M HCl containing 0.2% 2-mercaptoethanol at 110°C for 48 h. Cysteine was determined as cysteic acid in a separate analysis after performic acid oxidation.

RESULTS

When GTF-S was cleaved with trypsin, mouse submaxillaris protease, or lysyl endopeptidase, a large peptide fragment remained. Protease concentrations ranging from 1:100 to 1:100,000 (wt/wt) cleaved most of the protein to small peptides, but one large fragment consistently resisted digestion (Fig. 1). The three proteases have similar specificities: trypsin cleaves at lysyl and arginyl residues, lysyl endopeptidase cleaves at lysyl residues, and mouse submaxillaris protease cleaves at arginyl residues. Even though the protease-resistant fragment contained a high percentage of lysine and arginine (see amino acid composition), these residues were apparently protected by the protein tertiary structure. Disrupting GTF noncovalent structure by boiling for 10 min followed by trypsin digestion (1:5,000 [wt/wt]) for 20 h left no evidence of the trypsin-resistant fragment.

Mild proteolysis with proteases with specificities different from trypsin did not produce as simple a digestion pattern. Chymotrypsin (with primary specificity for aromatic amino acid residues) and *S. aureus* V8 protease (with specificity for acidic amino acid residues) gave distinct digestion patterns containing a more heterogeneous distribution of large peptides than was obtained by trypsin digestion (Fig. 2). GTF-I trypsin cleavage also had a rather complex digestion pattern (Fig. 3) compared with that of GTF-S. We subsequently found that a number of the larger GTF-I peptides bound dextran (data not shown). Nonetheless, the similarities among these fragments would make purification of a single

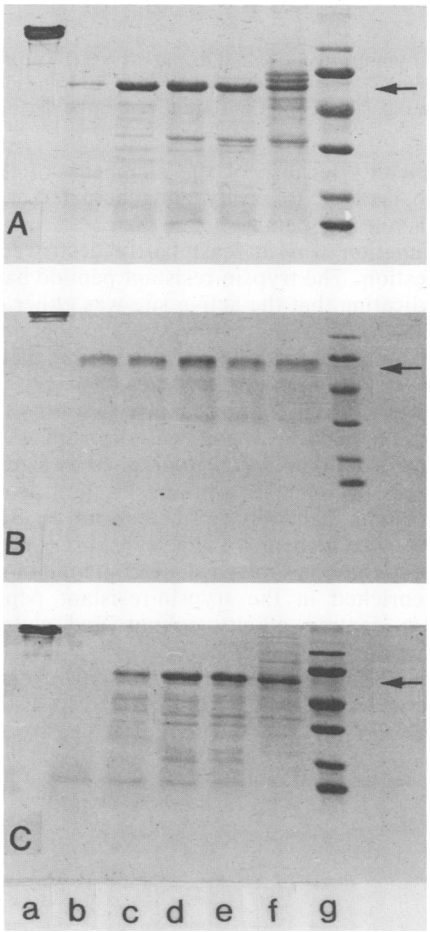

FIG. 1. Protease digests of GTF-S analyzed by SDS-PAGE (12% acrylamide). Proteases used were TPCK-trypsin (A), mouse submaxillaris protease (B), and lysyl endopeptidase (C). Lane a is native enzyme. Lanes b through f are 20-h, 37°C protease digests with protease/GTF-S ratios (wt/wt) of 1:100, 1:1,000, 1:5,000, 1:10,000, and 1:100,000, respectively. Lane g has molecular mass markers of 94, 67, 43, 30, 20.1, and 14.4 kDa. Gels are stained with Coomassie blue. The arrow indicates the peptide fragment which was purified.

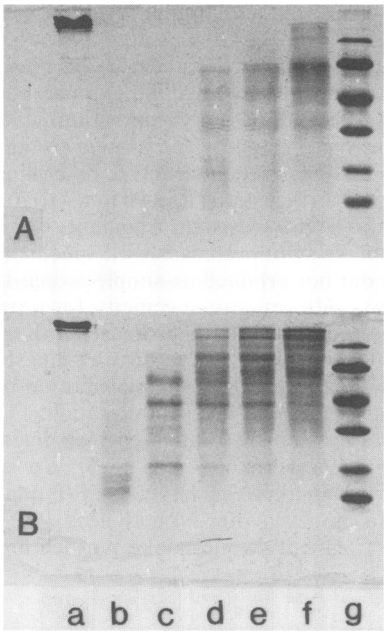

FIG. 2. Chymotrypsin (A) and *S. aureus* V8 (B) protease cleavage products of GTF-S analyzed by SDS-PAGE. Lane identification and protease digestion conditions are given in the legend to Fig. 1.

peptide difficult. Because of the simplicity of the GTF-S pattern, we decided to purify and characterize the large trypsin-resistant fragment.

GTF-S function was at least partly destroyed by mild trypsin digestion. The trypsin-resistant peptide had no GTF activity, indicating that the active site was either denatured or cleaved from the protein. However, the peptide bound to a dextran-based affinity column, suggesting that dextran-binding capacity was intact.

We used the dextran-binding capacity to purify the GTF-S tryptic peptide. GTF-S (1 mg) was digested with 1:5,000 (wt/wt) trypsin and then chromatographed by size exclusion HPLC (Fig. 4). In some preparations, fractions from the size exclusion column had only a single band on SDS-PAGE which migrated coincident with the trypsin-resistant peptide, and other preparations showed slight contamination (Fig. 5). Fractions enriched in the trypsin-resistant peptide were loaded on a dextran affinity column and eluted with a GndHCl gradient.

The glucan-binding peptide and the native enzyme had comparable dextran affinities: both eluted from the affinity column at nearly identical GndHCl concentrations (Fig. 6). We have previously reported that the affinity column is sensitive to protein-dextran affinities, since the GndHCl concentration required to elute GTF-S from the affinity column is lower than that required to elute GTF-I (18). The rather steep GndHCl gradient used to purify the glucan-binding peptide does not optimize detection of small differences in dextran binding. GndHCl elutes the protein from the affinity resin by disrupting glucan-protein bonds and/or reversibly denaturing the protein. In this experiment, it was not possible to determine the degree to which each factor was involved. Nonetheless, the similar elution profile of the glucan-binding peptide and its precursor indicated that the functional character of the peptide is relatively unaffected by loss of the majority of the protein molecule.

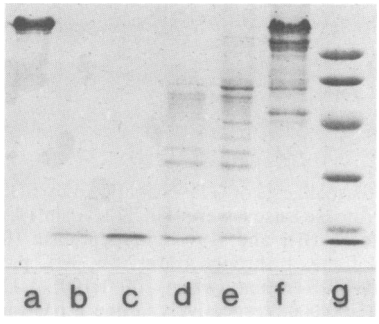

FIG. 3. Trypsin cleavage products of GTF-I analyzed by SDS-PAGE. Lane identification and conditions of protease digestion are given in the legend to Fig. 1.

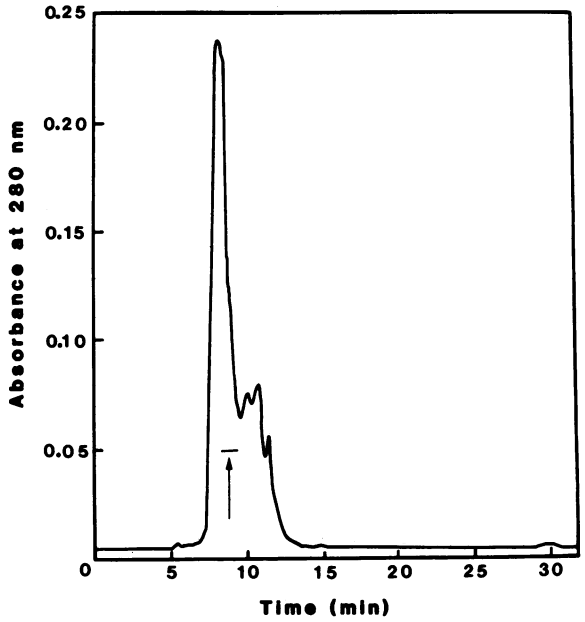

FIG. 4. Size exclusion HPLC of a 20-h, 1:5,000 (wt/wt) TPCK-trypsin digest of 1.0 mg of GTF-S. The TSK-250 column was eluted with 0.05 M sodium phosphate (pH 7.0) at a rate of 1 ml/min. The horizontal bar shows the fraction further purified by affinity chromatography. An SDS-PAGE analysis of the fraction is shown in Fig. 5.

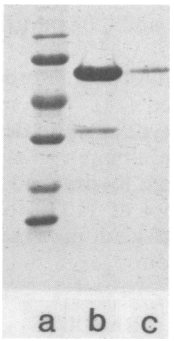

FIG. 5. SDS-PAGE analysis of the purity of the glucan-binding peptide after size exclusion HPLC and affinity chromatography. The glucan-binding peptide was purified from a 1:5,000 (wt/wt) trypsin digest of GTF-S. Lane a shows molecular weight markers (see legend to Fig. 1); lane b is a size exclusion HPLC fraction indicated in Fig. 4; and lane c is a peak eluting at approximately 2.0 M GndHCl from a dextran-based affinity column.

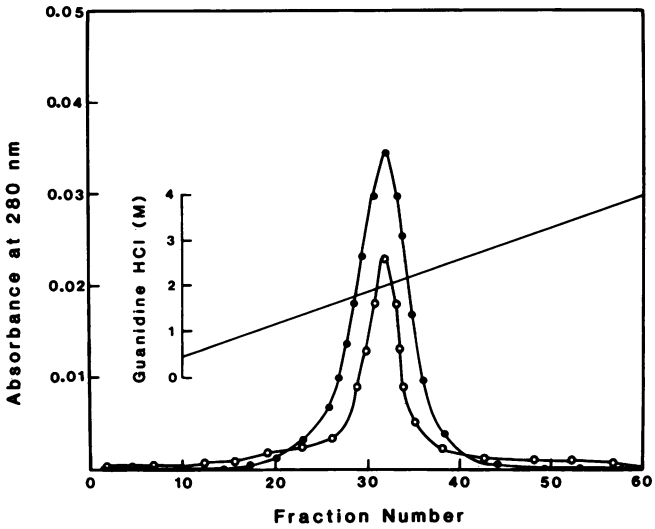

FIG. 6. Comparison of the elution profiles of the glucan-binding peptide and native GTF-S chromatographed on a dextran-based affinity column. After either 300 μg of GTF-S (●) or 200 μg of glucan-binding peptide (○) was loaded, the column was equilibrated with 0.05 M GndHCl in 0.1 M sodium phosphate buffer (pH 7.0) and then eluted with a 0.5 to 4.0 M GndHCl gradient at a slope of 0.067 M GndHCl per min and a flow rate of 1 ml/min.

Molecular weight calculations showed that the glucan-binding peptide represents about one-third the mass (60.5 kDa) (Fig. 7) of intact GTF-S (160 kDa). The amino acid compositions of GTF-S and the glucan-binding peptide were very similar; native GTF-S had slightly higher moles percent of hydrophilic residues (particularly aspartic acid-asparagine and glycine) and somewhat lower moles percent of hydrophobic residues (particularly valine and leucine) (Table 1).

TABLE 1. Amino acid composition of glucan-binding peptide and native GTF-S from *S. sobrinus*

Amino acid	mol% amino acid in:	
	Dextran-binding peptide	GTF-S
Half-cystine	0.0	0.0
Asparagine/aspartate	20.2	12.8
Threonine	7.4	8.9
Serine	6.0	7.9
Glutamine/glutamate	10.1	11.5
Proline	2.2	2.7
Glycine	13.2	9.8
Alanine	7.8	9.8
Valine	8.9	6.1
Methionine	.9	1.0
Isoleucine	2.1	3.3
Leucine	3.0	6.0
Tyrosine	8.8	7.8
Phenylalanine	4.9	3.9
Histidine	1.0	1.7
Lysine	5.9	5.0
Arginine	1.7	2.3

DISCUSSION

Large proteins are organized in domains, in part because this aids protein three-dimensional assembly during protein synthesis. Protein folding is more manageable when it occurs in discrete segments, which minimizes thermodynamic and steric complexities of assembling a large random structure (27). There are evolutionary advantages to domain structure as well. Protein segments which can maintain native form and function independently of external stabilizing forces have fewer structural demands when incorporated into a new protein through recombination. In part because of this, domain homologies are common in related proteins (14); such evolutionary sharing may occur in oral streptococcal GTFs as well.

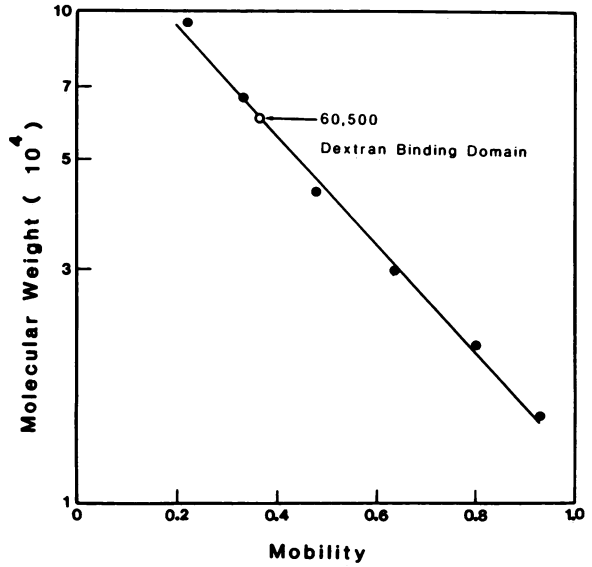

FIG. 7. Molecular weight analysis of the glucan-binding peptide. Analysis is based on relative electrophoretic mobility on 12% SDS-PAGE. Molecular weight standards are those listed in Fig. 1.

The glucan-binding peptide described here has characteristics consistent with a structural and functional domain of GTF-S. First, the peptide is relatively easily cleaved from the native protein with trypsin and trypsinlike proteases. Since some structural domains are large globular structures, not unlike subunits connected within the protein by short peptide chains (27), fortuitous susceptibility of the connecting chain to proteolysis can release the domain intact, as appears to have occurred with the glucan-binding segment of GTF-S. Second, the glucan-binding peptide is independently stable after removal of nearly two-thirds of the protein. The importance of the tertiary structure is clear from the observation that after heat denaturation, the glucan-binding peptide no longer resisted mild proteolysis. Finally, the peptide appears to be not only a structural domain, but a functional domain as well, with an affinity for dextran comparable to that of the native enzyme.

The presence of a glucan-binding domain as part of the GTF structure could account for some characteristics of GTF catalysis, such as the absence of a detectable reverse reaction and high sucrose specificity. Separate protein functions (like substrate-binding sites on multisubstrate enzymes) are often located on separate domains (6, 14). There is a growing body of data on a few two-substrate enzymes (referred to as hinge proteins) in which the position of the domains is dependent on substrate binding. In the absence of substrate, substrate-binding domains are not in correct alignment for catalysis and the enzyme is inactive; substrate binding induces a conformational realignment which brings the domains together to form the active catalytic site (1, 3, 9, 17).

Domain-related substrate-induced conformational changes may be involved in GTF catalysis as well. Kinetic studies have implicated substrate-induced conformational changes: sucrose binding increases enzyme affinity for dextran (18). If, in addition, sucrose binding is required to form the active catalytic site, this could account for the absence of a detectable reverse reaction, since sucrose is not present in the reverse reaction.

A hinge mechanism (or related substrate-induced conformational change) could also contribute to the high sucrose specificity of GTF. Sucrose substitutes are extremely rare despite numerous attempts to find high-affinity analogs that are either strong GTF inhibitors or alternative substrates (4, 5, 10, 25, and references therein). Substrate-induced active site formation imparts more strict specificity requirements than substrate binding alone.

Even though trypsin and trypsinlike protease cleavage of GTF-S produced a distribution of peptides highly enriched in one glucan-binding peptide, other protease digestions of GTF-S and GTF-I had a more heterogeneous distribution of large peptides. On the basis of preliminary data, a number of these peptides appeared to bind dextran. Recently, there have been several reports of glucan-binding receptors isolated from *S. mutans* culture broth and cell surfaces (16, 21, 23; D. Drake, K. J. Taylor, and R. J. Doyle, Annu. Meet. Int. Assoc. Dent. Res. 1986, abstr. 955, p. 226). At least one of these is a unique gene product (22), but not necessarily the others. Given that proteases are commonly found in *S. mutans* cultures and that GTF-S is susceptible to cleavage by these proteases (2, 8, 19), some of the putative glucan-receptor proteins may in fact be glucan-binding peptides derived from GTF.

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